WHOLE GENOME ANALYSIS OF TWO MULTIRESISTANT *Escherichia coli* STRAINS. EFFECT OF GRAPE POLYPHENOLS ON BACTERIAL GROWTH

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Introduction

The inappropriate use of antibiotics and their overuse has led to an increase of bacterial resistance to antibiotics. In the last few decades increasing rates of antibiotic resistance throughout the world have brought to light that resistance to antibiotics is currently a worrying issue of public health concern. The lack of effective antimicrobials to combat multi-resistant infection has exacerbated the situation and new alternatives are needed to tackle this problem.

Escherichia coli included in the common intestinal microbiota of humans and animals has become a useful indicator of the dissemination of antibiotic resistance in bacterial populations. Extended spectrum-betalactamases (ESBLs), which are the mayor cause to confer resistance to β-lactam antibiotics, are one of the most frequently found resistance mechanisms in *E. coli* (Poirel *et al.* 2018). ESBLs are often found in plasmids (de Toro *et al.* 2014) and therefore, associated to other antibiotic resistance genes and related with cross-infection (Jesumirhewe *et al.* 2020; Zango *et al.* 2019).

The benefits of natural products of vegetable origin are widely known, and they have been used for multiple applications, including empirical treatments of infections, for centuries without generating bacterial resistance. Polyphenols of plant extracts possess a significant antioxidant and antibacterial activity (Bouarab Chibane *et al.* 2019; Luchian *et al.* 2019). *Vitis vinifera* red grapes contain polyphenols mainly in the solid parts of the berry (skins and seeds), and they are classified in flavonoid (anthocyanidins, flavonols, flavan-3-ol or proanthocyanidins) and non-flavonoid compounds (Quijada-Morín *et al.* 2015). Graciano is a red variety of *Vitis vinifera* cultivated traditionally in the northern Spanish region of Rioja, and it is positively valued because of its high anthocyanin content (Núñez *et al.* 2014) and its contribution to improving wine colour intensity.

The antimicrobial activity of polyphenols has been attributed to a variety of mechanisms: interaction with bacterial cell wall and membrane, inhibition of biofilm formation, inhibition of bacterial enzymes, and protein regulation (Daglia *et al.* 2012; Papuc *et al*. 2017). Among polyphenols, flavonoids have been object of a number of studies and have been reported as antimicrobial agents against gram positive and negative bacteria, and their action has been associated with their structure (Darra *et al.* 2012; Farhadi *et al.* 2019; Górniak *et al.* 2019; Rodríguez-Pérez *et al*. 2016).

This study aimed to investigate the resistome of two multidrug resistant *E. coli* strains isolated from the natural intestinal microbiota of healthy animals, and the effect of a red grape polyphenol extract from Graciano grape skins on the growth of these multidrug resistant *E. coli* strains.

Methods

1. Bacterial strains

Two multidrug resistant *E. coli* strains C6898 and C6840 of intestinal origin, isolated from chicken and coati respectively, were used for this study. Both strains belong to the strain collection of the University of La Rioja, their resistance phenotypes and genotypes had been analyzed, and they were assigned the ESBL genotype.

2. Genome analysis of the multiresistant strains

Total DNA extraction of both *E. coli* strains C6898 and C6840 was performed with the QIAmp DNA Mini Kit (Qiagen GmbH, Qiagen Hilden, Germany). Libraries were prepared by using the TruSeq DNA PCR-Free protocol (Illumina, San Diego, California, United States) at the Genomics and Bioinformatics Core Facility (Centre for Biomedical Research of La Rioja). Paired-end 100-bp reads on fragments of 550-bp insert size were sequenced in an Illumina HiSeq 1500. Genomes were reconstructed by using PLACNETw (Vielva *et al.* 2017). Identification of Open Reading Frames (ORFs) and genome annotation of the assembled genetic elements was performed by using Prokka (Seemann, 2014).

Specific multi-locus sequence typing (MLST) analysis of the *E. coli* strains was performed automatically *in silico* on the basis of the following genes: *adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A and *rec*A. The MLST typer (Larsen *et al.* 2012) database was used. Three different databases were used for the identification of antibiotic resistance genes: ResFinder (Zankari *et al.* 2012), CARD Resistance Gene Identifier (McArthur et al., 2013), and ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) (Gupta *et al.* 2014).

3. Polyphenol extract from grape skins

The polyphenol extract was obtained from the skins of *Vitis vinifera* L. cv. Graciano grapes of the Appellation of Origin Rioja, and it was kindly provided by the Polyphenol Research Group (GIP-USAL) of the University of Salamanca. Table 1 shows the phenolic composition of the extract.

Table 1. Chemical composition of the extract of Graciano grape skins

Polyphenol	Content (mg / g of extract)
TOTAL Flavonols	162,9 ± 3,8
Myricetin	97,5 ± 2,0
Quercetin	36,4 ± 0,6
Kaempferol	29,0 ± 1,3
TOTAL Antocyanins	402,5 ± 12,6
Delphinidin	64,8 ± 1,2
Cyanidin	32,1 ± 1,7
Petunidin	63,7 ± 1,7
Peonidin	97,6 ± 3,6
Malvidin	144,3 ± 4,4

4. Evaluation of the antimicrobial activities

The antibacterial activities of the Graciano grape skin extract (GG), the pure polyphenols: malvidin and epicatechin (Extrasynthese, Lyon, France), and the reference antibiotic ampicillin (Sigma Chemical Co., Germany) were tested against both *E. coli* strains C6898 and C6840. The microtiter dilution assay, using serial double dilutions of the antimicrobial agent (Rojo-Bezares *et al*, 2007), was used to determine the minimal inhibitory concentration (MIC) of the antimicrobial agents. Strains were incubated in BHI broth

(Oxoid, Thermo Fisher) at 30ºC for 24 h in the microplate reader Tecan Spark 10M (Tecan Trading AG, Switzerland) and optical density at 600 nm was measured every 30 min, which provided bacterial growth curves and MIC values. Controls were included in all the assays. GG was tested in the concentration range from 6.25 mg/mL to 0.1 mg/mL, malvidin and epicatechin from 1.33 mg/mL to 0.04 mg/mL, and ampicillin from 4.19 mg/mL to 0.13 mg/mL. Assays combining GG and ampicillin were performed for both *E. coli* C6898 and C6840 strains to study putative synergistic effects. The checkerboard method (Cantón *et al.* 2000) was used to determine the fractional inhibitory concentration (FIC) index.

Results and Discussion

1. Genome analysis

Results of the genome analysis of both *E. coli* strains C6898 and C6840 are shown in Table 2. Strain C6840 showed one unique plasmid and its chromosome was larger than that of C6898, whereas this later strain showed three different plasmids.

Table 2: Genomes of *E. coli* strains C6898 and C6840

Strain	Genome element	Size (bp)	MLST type
C6840	chromosome C6840	4907211	ST949
	plasmid 1	92322	
C6898	chromosome C6898	4794736	ST746
	plasmid 1	74024	
	plasmid 2	108014	
	plasmid 3	132901	

2. Antibiotic resistance profiles

Strain C6840 showed 59 genes related to antibiotic resistances, two of which were located in its plasmid. Strain C6898 showed 48 genes related to antibiotic resistances in its chromosome, and 6 more resistance genes in each of its two plasmids. The resistance profiles obtained by whole genome sequencing were in agreement with the previous characterization performed with conventional methods, showing multidrug resistance for both strains. Genes encoding different efflux pumps, antibiotic modifying enzymes, which included beta-lactamase enzymes, were identified. Table 3 shows the resistance phenotypes and the multilocus sequence typing of both *E.coli* strains.

Table 3. Antibiotic resistance phenotypes of the *E. coli* strains

Name	Resistance phenotype	MLST
C6898	AMP, CTX, TET, SXT	ST746
C6840	AMP, CTX, NAX, TET, SXT	ST949

AMP: ampicillin; CTX: cefotaxime; NAX: nalidixic acid; TET: tetracycline and SXT: sulfametoxazole

3. Antimicrobial activity of the Graciano grape extract

The effect of GG on the growth of both strains C6840 and C6898 was tested in concentrations from 6.25 to 0.1 mg/mL, and the concentration of GG 6.25 mg/mL was able to reduce bacterial population down to 50% with respect to the control assay without GG. Bacterial growth of both strains was totally inhibited in presence of 12.5 mg/mL of GG (MIC = 12.5 mg/mL) (Figure 1), and this effect was bacteriostatic.

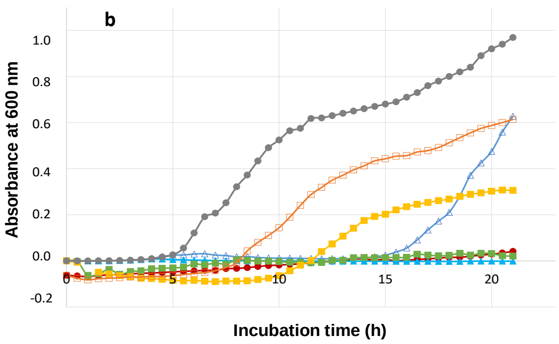
4. Synergistic effect of GG on the growth of the multiresistant stains

As expected from the results of the genome analysis, both *E. coli* strains C6898 and C6840 showed high resistance (MIC values ≥ 3 mg/mL) to the antibiotic of reference AMP according to the CLSI (Clinical and Laboratory Standards Institute), and thus, AMP MIC was ≥4.19 and 3.06 mg/mL for C6898 and C6840 respectively.

The experiments combining GG and AMP by the checkerboard method were performed to calculate the fractional inhibitory concentration (FIC) index, considering the lowest FIC value without bacterial growth. The FIC values indicate the effect of the combination: FIC ≤0.5 synergy, 0.5≤FIC≤1 partial synergy; 1≤FIC≤2 addition and FIC≥2 antagonism (Cantón *et al.* 2000).

A combination of 1.56 mg/mL GG with a subinhibitory concentration of AMP (2.25 mg/mL) totally inhibited the growth of the multi-resistant *E. coli* strain C6898, and presented a FIC value of 0.4 of total synergy. Similarly, the combination of 3.13 mg/mL GG with a subinhibitory concentration of AMP (1.13 mg/mL) totally inhibited the growth of the multi-resistant *E. coli* strain C6840, and presented a FIC value of 0.6 of partial synergy between both agents (Figure 1).

a



(a) C6898
12.5 mg/ml GG
6.25 mg/ml GG
1.56 mg/ml GG
4.19 mg/ml AMP
2.25 mg/ml AMP
1.56mg/ml GG + 2.25mg/ml AMP
control (BHI)

(b) C6840
12.5 mg/ml GG
6.25 mg/ml GG
3.13 mg/ml GG
3.06 mg/ml AMP
1.13 mg/ml AMP
3.13mg/ml GG+1.13mg/ml AMP
control (BHI)

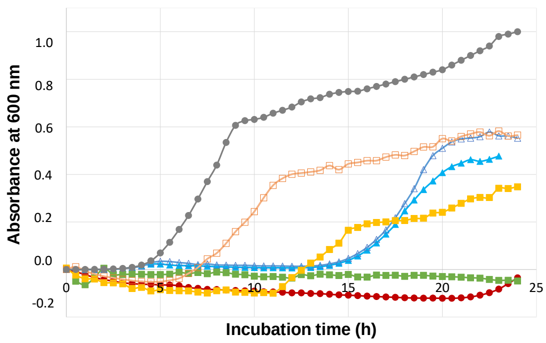


Figure 1. Growth curves for the *E. coli* strains C6898 (a) y C6840 (b) with

These results indicate that the presence of the polyphenolic extract of Graciano grape skins turned both multiresistant *E. coli* strains sensitive to a small concentration of the β-lactam antibiotic. Some pure polyphenols have been reported to show synergy with antibiotics, that is the case of quercetin and tetracycline against *E. coli* (Qu *et al.* 2019), quercetin and kaempferol combined with rifampicin against *Sthapylococcus aureus*, and epigallocatechin gallate with oxacillin (Daglia *et al*. 2012).

Our results show that not only pure polyphenols, but an extract obtained from grape skins can be effectively used to turn sensitive multi-drug resistant *E. coli* strains.

4. Malvidin and epicatechin effect on bacterial growth

Epicatechin and malvidin were chosen as representative polyphenols of both flavanol and anthocyanin families. They were assayed against strain C6840 that showed the highest number of antibiotic resistance genes in its bacterial chromosome. Both polyphenols were assayed in concentrations from 1.33 to 0.04 mg/mL and both showed bacterial growth inhibition (Figure 2), they totally controlled bacterial population with a MIC = 0.67 mg/mL for both compounds. Moreover, 1.33 mg/mL of malvidin showed bactericide effect on this multidrug resistant *E. coli* strain, whereas epicatechin showed bacterostatic effect.

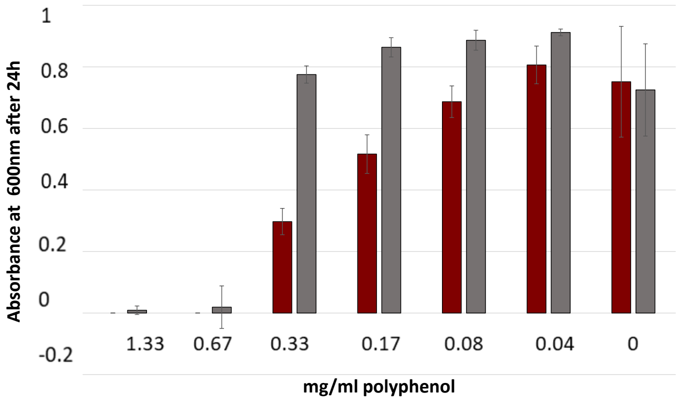


Figure 2. Effect of polyphenol concentration on the growth of *E. coli* C6840
■ malvidin and ■ epicatechin

These results show that malvidin, which was the most abundant anthocyanin in GG, presented a potent inhibitory effect on the growth of the multiresistant *E. coli* strain. These results are in concordance with reports of antimicrobial activity of oenological products against pathogenic and non-pathogenic microorganisms (Luchian *et al*. 2019; Górniak *et al.* 2019) and with the reported effect of anthocianydins and flavonols on *E. coli* (Daglia *et al.* 2012; Trikas *et al.* 2016).

Summarizing, our study shows that a natural polyphenol extract obtained from Graciano grape skins can be used to control the growth of multiresistant *E. coli* strains and that grape skin polyphenol extracts could contribute to reduce the current prophylactic and therapeutic use of antibiotics, and thus contribute to combat widespread antibiotic resistance.

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